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Applicants	:	Qiu et al.	Examiner: A. Kubelik
Serial No.	:	09/766,348	
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For	:	HYPERSENSITIVE RESPONSE INDUCED RESISTANCE IN PLANTS BY SEED TREATMENT	

DECLARATION OF ZHONG-MIN WEI UNDER 37 C.F.R. § 1.132

U.S. Patent and Trademark Office
P.O. Box 2327
Arlington, VA 22202

Dear Sir:

I, ZHONG-MIN WEI, pursuant of 37 C.F.R. § 1.132, declare:

1. I received a B.S. degree in Biology from Zhejiang University, Zhejiang, China in 1982, an M.S. degree in Plant Pathology from Nanjing Agricultural University, Nanjing, China in 1984, and a Ph.D. degree in Molecular Biology from Nanjing Agricultural University and Academy of Science, Shanghai, China in 1987.

2. I am currently employed as Chief Scientific Officer and Vice President of Research and Development at EDEN Bioscience Corporation in Bothell, Washington.

3. I am an inventor of the above-identified application.

4. I am presenting this declaration to show that hypersensitive response elicitors from a diverse range of plant pathogenic bacteria (1) are an art-recognized class of proteins where results achieved with one such protein would be expected when other proteins in this class are used and (2) share the unique ability to cause distinct plant responses. Specifically, treatment of a variety of plants and plant seeds with hypersensitive response elicitors was shown to induce plant disease resistance, enhance plant growth, and induce

plant stress resistance, as compared with plants and plant seeds not treated with a hypersensitive response elicitor.

5. In plants, the hypersensitive response phenomenon results from an incompatible interaction between plant pathogens and non-host plants. As explained in Gopalan et al., "Bacterial Genes Involved in the Elicitation of Hypersensitive Response and Pathogenesis," Plant Disease 80: 604-10 (1996) ("Gopalan") (attached hereto as **Exhibit 1**), these types of interactions involve, for example, a bacterial plant pathogen attempting to infect a host plant, and the host plant preventing proliferation of the pathogen by the collapse and death, or necrosis, of plant leaf cells at the site of infection. This is distinct from a compatible interaction between a bacterial plant pathogen and a host plant in which the bacteria is capable of proliferation, resulting in the spread of the pathogen throughout the plant and the manifestation of disease symptoms. Id. at 604.

6. Hypersensitive response elicitors within a given genus are often homologous to elicitors from different pathogenic species and strains of the same genus. For example, homologs of hypersensitive response elicitors from *Erwinia amylovora* and *Pseudomonas syringae* have been identified in different bacteria species and strains from the genera *Erwinia* and *Pseudomonas*, respectively. See Gopalan.

7. In addition, numerous reported studies confirm that a gene encoding a hypersensitive response elicitor from a particular source genus can be used to isolate a corresponding hypersensitive response elicitor gene from different species and strains of that same genus. For example, in Bauer et al., "*Erwinia chrysanthemi* Harpin_{Ech}: An Elicitor of the Hypersensitive Response that Contributes to Soft-Rot Pathogenesis," MPMI 8(4): 484-91 (1995) ("Bauer") (attached hereto as **Exhibit 2**), the *Erwinia amylovora* hypersensitive response elicitor encoding gene was used as a probe to isolate, clone, and sequence the gene encoding the *Erwinia chrysanthemi* hypersensitive response elicitor, as follows:

> The cosmids were probed in colony blots with a 1.3-kb *Hind*III fragment from pCPP1084, which contains the *E. amylovora hrpN* gene (Wei et al. [, "Harpin Elicitor of the Hypersensitive Response Produced by the Plant Pathogen *Erwinia amylovora*," Science 257:85-88 (]1992[)]). pCPP2157, one of the three cosmids hybridizing with the probe, was digested with several restriction enzymes, and the location of the $hrpN_{Ech}$ gene in those fragments was determined by probing a Southern blot with *E. amylovora Hind*III fragment. Two fragments, each containing the entire $hrpN_{Ech}$ gene, were subcloned into different vectors: pCPP2142 contained an 8.3-kb *Sal*I fragment in pUC119 (Vieira and Messing [,"Production of Single-Stranded Plasmid DNA," Methods Enzymol., 153:3-11(]

> 1987[])], and pCPP2141 contained a 3.1-kb *Pst*I fragment in pBluescript II SK(-) (Stratagene, La Jolla, CA).
>
> ***Sequence of hrpN$_{Ech}$***
>
> The nucleotide sequence of a 2.4-kb region of pCPP2141 encompassing *hrpN$_{Ech}$* was determined. The portion of that sequence extending from the putative ribosome-binding site through the *hrpN$_{Ech}$* coding sequence to a putative rho-independent terminator is presented in Figure 1.

See page 485.

8. In the same manner as described in Bauer *supra*, Cui et al., "The RsmA$^-$ Mutants of *Erwinia carotovora* subsp. *carotovora* Strain Ecc71 Overexpress *hrp*N$_{Ecc}$ and Elicit a Hypersensitive Reaction-like Response in Tobacco Leaves," MPMI 9(7): 565-73 (1996) ("Cui") (attached hereto as **Exhibit 3**) demonstrates that the gene encoding the *Erwinia carotovora* hypersensitive response elicitor can be isolated, sequenced, and cloned by using the *Erwinia chrysanthemi* hypersensitive response elicitor encoding gene to probe the genomic library of *Erwinia carotovora*. Further, Cui (at page 572) states the following:

> The genomic library of *E. carotovora* subsp. *carotovora* strain Ecc71 in pLARF5 was screened by in situ colony hybridization with a 0.75-kb internal *Cla*I fragment of *hrpN* of *E. chrysanthemi* (Bauer et al.[, "*Erwinia chrysanthemi* Harpin$_{Ech}$: An Elicitor of the Hypersensitive Response that Contributes to Soft-Rot Pathogenesis," MPMI 8(4): 484-91 (]1995). Two cosmids, pAKC921 and pAKC922, that hybridized with the probe were isolated. The subclones (pAKC923 and pAKC924, Table 1) carrying *hrpN* DNA were used for sequence analysis.

9. The gene encoding the hypersensitive response elicitor of *Erwinia amylovora* has also been used as a probe to isolate and clone the gene encoding the hypersensitive response elicitor of *Erwinia stewartii*. It was found that antibodies raised against the hypersensitive response elicitor of *Erwinia stewartii* cross-reacted with the hypersensitive response elicitor of *Erwinia amylovora*. See Ahmad et al., "Harpin Is Not Necessary for the Pathogenicity of Maize," 8th Int'l Cong. Molec. Plant Microbe Inter. July 14-19, 1996 ("Ahmad") (attached hereto as **Exhibit 4**).

10. Similar findings were reported for hypersensitive response elicitors from the genus *Pseudomonas*. An internal fragment of the hypersensitive response elicitor from *Pseudomonas syringae* pv. *syringae* (i.e., *hrpZ*) was used to identify and isolate the hypersensitive response elicitors from *P. syringae* pv. *glycinea* and *P. syringae* pv. *tomato*.

Significant amino acid sequence similarities were identified between the various *Pseudomonas syringae* elicitors. See Preston et al., "The HrpZ Proteins of *Pseudomonas syringae* pvs. *syringae, glycinea,* and *tomato* Are Encoded by an Operon Containing *Yersinia ysc* Homologs and Elicit the Hypersensitive Response in Tomato But Not Soybean," MPMI 8(5): 717-32 (1995) ("Preston") (attached hereto as **Exhibit 5**).

11. The genes encoding hypersensitive response elicitors are positioned within the *hrp* gene cluster or proximate to the *hrp* gene cluster in *hrp* regulons. For example, *hrpN* from *Erwinia amylovora* was located within the *hrp* gene cluster, as was *hrpZ* from *Pseudomonas syringae*. The *popA* gene, encoding a hypersensitive response elicitor from *Pseudomonas solanacearum*, was located on the left flank of the *hrp* gene cluster within a *hrp* regulon. See Bonas, "*hrp* Genes of Phytopathogneic Bacteria," Current Topics in Microbiology and Immunology 192: 79-98 (1994) ("Bonas I") (attached hereto as **Exhibit 6**) and Alfano et al., "The Type III (Hrp) Secretion Pathway of Plant Pathogenic Bacteria: Trafficking Harpins, Avr Proteins, and Death," Journal of Bacteriology 179: 5655-5662 (1997) ("Alfano") (attached hereto as **Exhibit 7**). Similar to the *popA* gene, *hreX*, the gene encoding the hypersensitive response elicitor from *Xanthomonas campestris*, was located on the left flank of the *hrp* gene cluster. See Swanson et al., "Isolation of the *hreX* Gene Encoding the HR Elicitor Harpin (Xcp) from *Xanthomonas campestris* pv. *pelargonii*," Phytopathology 90: s75 (1999) ("Swanson") (attached hereto as **Exhibit 8**).

12. The characteristics that distinguish hypersensitive response elicitors as a distinct class of molecules are clearly apparent when considering the different elicitors' secretion mechanisms, regulation, biochemical characteristics, and biological activities.

13. Substantially all hypersensitive response elicitors identified have been shown to be secreted through the type III, *hrp* dependent secretion pathway. The type III secretion pathway is a highly conserved and unique mechanism for the delivery of pathogenicity related molecules in gram-negative bacteria. The *hrp* gene cluster is largely composed of components of the type III secretion system. See Bogdanove et al., "Unified Nomenclature for Broadly Conserved *hrp* Genes of Phytopathogenic Bacteria," Molec. Microbiol. 20:681-83 (1996) ("Bogdanove") (attached hereto as **Exhibit 9**); and Alfano.

14. Regulation of the genes encoding the *hrp* gene cluster, and subsequently the genes encoding the components of the type III secretion system and hypersensitive response elicitors, is controlled by environmental factors. Specifically, transcriptional expression of these genes is induced under conditions that mimic the plant apoplast, such as low concentrations of carbon and nitrogen, low temperature, and low pH.

See Wei et al., "Regulation of hrp Genes and Type III Protein Secretion in *Erwinia amylovora* by HrpX/HrpY, a Novel Two-Component System, and HrpS," MPMI 13(11): 1251-1262 (2000) ("Wei I") (attached hereto as **Exhibit 10**); and Bonas I.

15. Biochemically, hypersensitive response elicitors have a number of common characteristics. These include being glycine rich, heat stable, hydrophilic, lacking of an N-terminal signal sequence, and susceptible to proteolysis. See Bonas, "Bacterial Home Goal by Harpins," Trends Microbiol 2: 1-2 (1994) ("Bonas II") (attached hereto as **Exhibit 11**); Bonas I; Gopalan; and Alfano.

16. In addition, hypersensitive response elicitors share a unique secondary structure that has been associated with these elicitors' distinct biological activities (described below). The structure has two primary components, an alpha helix unit and a relaxed acidic unit having a sheet or random turn structure. In the absence of one or both of these components, hypersensitive response elicitation does not occur. See WO 01/98501 to Fan et al. ("Fan") (attached hereto as **Exhibit 12**).

17. In addition to eliciting the hypersensitive response in a broad range of plant species, as explained by Wei et al., "Harpin from *Erwinia amylovora* Induced Plant Resistance," Acta Horticulture 411: 223-225 (1996) ("Wei II") (attached hereto as **Exhibit 13**) and by Alfano, hypersensitive response elicitors also share the ability to induce specific plant responses. The induction of plant disease resistance, plant growth enhancement, and plant stress resistance are three plant responses that result from treatment of plants or plant seeds with a hypersensitive response elicitor from a gram-negative plant pathogen.

18. As described in Wei II, treatment of plants with the hypersensitive response elicitor HrpN from *Erwinia amylovora* resulted in disease resistance to a broad range of plant pathogens. For example, HrpN induced disease resistance to southern bacterial wilt (*Pseudomonas solanacearum*) in tomato, tobacco mosaic virus in tobacco, and bacterial leaf spot (*Gliocladium cucurbitae*) in cucumber.

19. The hypersensitive response elicitor HrpZ from *Pseudomonas syringae* was reported to induce disease resistance in cucumber to a diverse range of pathogens, including the fungal disease *Colletotrichum lagenarium*, tobacco necrosis virus, and bacterial angular leaf spot (*Pseudomonas syringae* pv. *lachrymans*). See Strobel et al., "Induction of Systemic Acquired Resistance in Cucumber by *Pseudomonas syringae* pv. *syringae* 61 $HrpZ_{Pss}$ Protein," Plant Journal 9(4): 431-439 (1996) ("Strobel") (attached hereto as **Exhibit 14**).

20. Hypersensitive response elicitors from *Erwinia amylovora* and *Pseudomonas syringae* pv. *syringae* are also known to enhance plant growth. See Examples 1 to 24 of U.S. Patent No. 6,277,814 to Qiu et al. ("Qiu") (attached hereto as **Exhibit 15**), which showed that treatment of plants and plant seeds with HrpN from *E. amylovora* induced plant growth enhancement in species of tomato, potato, raspberry, and cucumber.

Hypersensitive Response Elicitors Induce Plant Disease Resistance

21. As demonstrated by the following experimental evidence in paragraphs 22 and 23 below, treatment of tomato and tobacco plants with the hypersensitive response elicitor HreX from *Xanthomonas campestris* pv. *pelargonii* induced disease resistance in the plants against bacterial wilt and tobacco mosaic virus.

22. The induction of disease resistance in tomato against bacterial wilt (caused by the pathogenic bacterium *Pseudomonas solanacearum* K_{60}) was investigated as follows. Approximately 30 days after sowing, tomato plants were sprayed with either a dilution of HreX or 5 mM potassium phosphate buffer, pH 6.8 (the same buffer used to dilute the HreX solution). Six days after treatment, inoculation was performed by slicing the soil of the pot containing the tomato plant 4 times and applying 40 ml of solution containing 1 x 10^6 colony forming units ("cfu") per ml of *P. solanacearum* K_{60} to the soil. Disease severity ratings were recorded at 7, 9, and 13 days after inoculation ("DAI"), as shown below in Table 1. As these results demonstrate, plants treated with HreX exhibited substantially more disease resistance than the buffer-treated control plants.

Table 1. ***Pseudomonas solanacearum*** **Disease Resistance from Treatment of Tomato with HreX.**

Treatment Groups[a]	Disease Index (7 DAI)	Disease Index (9 DAI)	Disease Index (12 DAI)	% Difference (12 DAI)
HreX	0.12	0.22	0.22	38.89
Buffer	0.16	0.3	0.36	na

[a]Each group consisted of 1 pot containing 10 plants.

23. Experiments examining the induction of systemic disease resistance in tobacco from treatment with HreX were conducted as follows: Diluted HreX was sprayed on all but the bottom most full-sized leaf of six- to eight-week-old tobacco plants (Xanthi). The bottom most full-sized leaf was covered during spraying so as not to receive residual spray. Three days after the spray treatment, the unsprayed leaf and the leaf opposite it, were lightly

dusted with diatomaceous earth. Thereafter, 20 µl of a 1.7 µg/ml solution of tobacco mosaic virus ("TMV") was applied to both leaves dusted with diatomaceous earth. The TMV was gently and evenly spread across the leaves. Approximately 5 minutes after inoculation, the plants were lightly rinsed to remove the diatomaceous earth. Three days after inoculation, the number of TMV lesions on the unsprayed and sprayed leaves for each plant was recorded, as shown below in Table 2. As these results demonstrate, plants treated with HreX exhibited substantially more disease resistance than the buffer-treated control plants.

Table 2. Tobacco Mosaic Virus Resistance in Tobacco from Treatment with HreX.

Treatment Groups	Number of TMV Lesions on Leaf									
	Treated leaves					Untreated leaves				
	Plant No. 1	Plant No. 2	Plant No. 3	Avg. No.	% Difference	Plant No. 1	Plant No. 2	Plant No. 3	Avg. No.	% Difference
HreX	5	7	8	6.67a	93.37	41	22	20	27.67a	76.49
Buffer Control	107	99	96	100.67b	na	124	106	123	117.67b	na

Enhanced Plant Growth by Treatment of Plants with Hypersensitive Response Elicitor

24. As demonstrated by the following experimental evidence in paragraphs 25 and 26 below, treatment of plants with hypersensitive response elicitors from a range of sources, such as *Psuedomonas syringae* (HrpZ) and *Xanthomonas campestris* (HreX), enhances plant growth.

25. The hypersensitive response elicitor HreX from *Xanthomonas campestris* was evaluated for induction of plant growth enhancement as follows: Prior to sowing, tomato seeds were soaked for approximately four hours in either a solution containing the partially purified HreX protein diluted in potassium-phosphate buffer, or potassium-phosphate buffer alone. The treated seeds were then planted and maintained in identical conditions in a controlled environment. Each treatment group consisted of 3 pots, each pot containing 8 plants. The average plant heights and percent differences between the treatment groups are shown below in Table 3. As these results demonstrate, plants treated with HreX grew significantly more than the buffer-treated control plants.

Table 3. Growth Enhancement from Treatment of Tomato with the Hypersensitive Response Elicitor HreX.

Treatment Groups	Replicates[1]			Mean[2]	% Difference
	Pot #1	Pot #2	Pot #3		
HreX	7.4	7.3	6.8	7.1a	15.5
Buffer Control	6.1	6.1	5.6	6.0b	na

[1] Mean height of the 8 plants in each pot.
[2] Means followed by the same letter do not significantly different (P=0.01, LSD)

26. The hypersensitive response elicitor HrpZ from *Psuedomonas syringae* was evaluated for induction of plant growth enhancement as follows: Prior to sowing, tomato seeds were soaked for approximately four hours in either a solution containing the partially purified HrpZ protein diluted in potassium-phosphate buffer, or potassium-phosphate buffer alone. The treated seeds were then planted and maintained in identical conditions in a controlled environment. Each treatment group consisted of 6 pots, each pot containing 10 plants. The average plant heights and percent differences between the treatment groups are shown below in Table 4. As these results demonstrate, plants treated with HrpZ grew significantly more than the buffer-treated control plants.

Table 4. Growth Enhancement from Treatment of Tomato with the Hypersensitive Response Elicitor HrpZ.

Treatment Groups	Replicates[1]						Mean[2]	% Difference
	Pot #1	Pot #2	Pot #3	Pot #4	Pot #5	Pot #6		
HrpZ	5.10	5.28	4.60	4.72	4.71	4.87	4.88a	9.6
Buffer Control	4.15	4.38	3.84	4.31	4.62	5.18	4.41b	na

[1] Mean height of the 18 to 21 plants in each pot.
[2] Means followed by the same letter do not significantly differ (P=0.054, LSD)

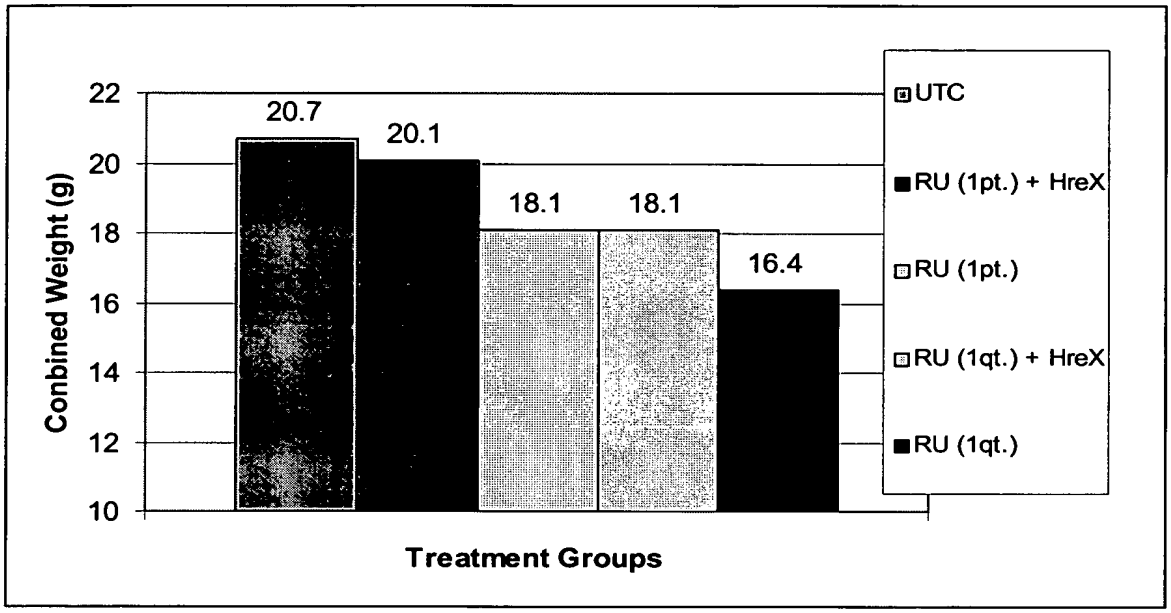
Hypersensitive Response Elicitors Induce Plant Stress Resistance

27. As evidenced by the experimental results reported in Examples 1-6 of WO 00/28055 to Wei et al. (attached hereto as **Exhibit 16)**, HrpN from *Erwinia amylovora* is capable of inducing various forms of plant stress resistance, such as chemical stress resistance, drought stress resistance, and nutritional stress resistance.

28. As demonstrated by the following experimental evidence in paragraphs 29 through 32 below, the hypersensitive response elicitor HreX from *Xanthomonas campestris* is also capable of inducing various forms of plant stress resistance, such as chemical stress resistance and salt stress resistance.

29. In order to investigate whether treatment of plants with the hypersensitive response elicitor HreX from *Xanthomonas campestris* induces chemical stress resistance, corn seeds (DK662RR) were treated with HreX and then treated with varying concentrations of Roundup® (active ingredient glyphosate, Monsanto Co., St. Louis, MO). The HreX treatments consisted of soaking the corn seeds in 100 ml of a solution containing a 3% formulation of HreX dissolved in water. The seeds were soaked for approximately 4 hours at 26°C. The corn seeds were sown in pots containing vermiculite and equal amounts of the fertilizer EcoGrow (ECO Enterprises, Shoreline, WA). Roundup® (RU) treatments were conducted by a single spraying of the corn seedlings approximately two weeks after germination. Roundup® was applied at two concentrations. At the 1 pint (1pt.) application rate, 4.73 ml of Roundup® was mixed with 189 ml water. At the 1 quart (1qt.) application rate, 9.46 ml of Roundup® was mixed with 189 ml of water. The specific treatment groups were as detailed below in Figure 1. Fifteen seeds were planted in each pot with a total of six pots per treatment. Plants were grown at 22°C to 26°C with a 14 hour daylight period. Results were obtained by measuring the dry weight from the largest 10 plants from each pot. The plants were dried by isolating the entire plant from the vermiculate and drying overnight at 26°C. The Combined Weight shown below in Figure 1 represents the accumulated dry weight of the 60 plants measured from each treatment group. The untreated control (UTC) plants were not pretreated with HreX and were not treated with Roundup®.

Figure 1. Dry Weight of Chemical + HreX and Chemical Alone Treated Plants

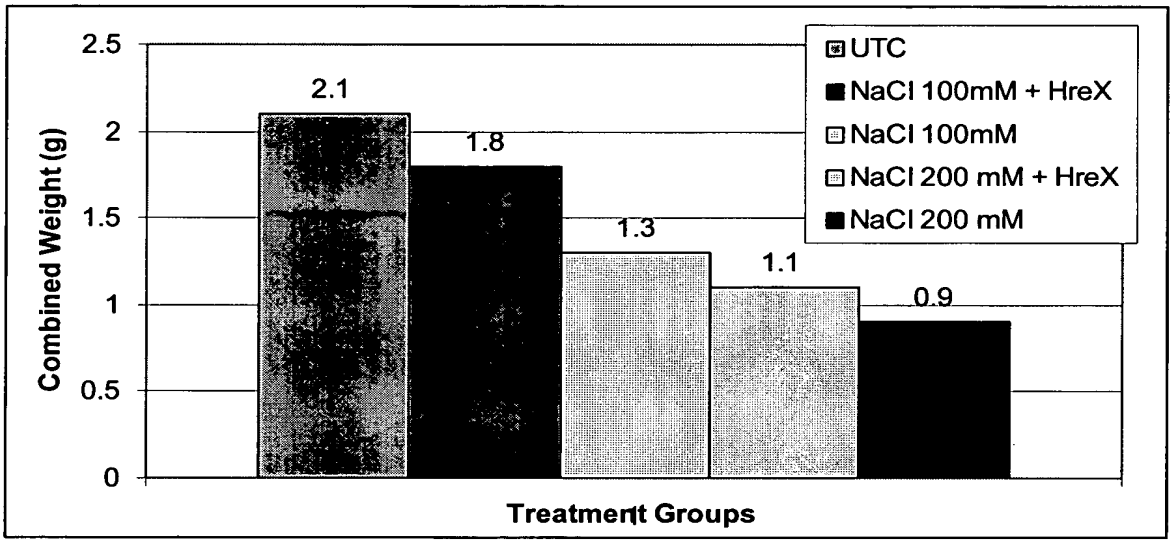


30. The hypersensitive response elicitor HreX clearly imparts chemical stress resistance in plants as demonstrated in Figure 1. Treatment of plants with Roundup® led to decreases in plant dry weight of approximately 13% at the Roundup® application rate of 1 pint, and approximately 21% at the Roundup® application rate of 1 quart, in comparison to that of the untreated control plants. In contrast, plants treated with HreX in combination with Roundup® resulted in decreases in dry weight of approximately 3% at the Roundup® application rate of 1 pint, and approximately 13% at the Roundup® application rate of 1 quart, in comparison to that of the untreated control plants. The treatment of plants with the hypersensitive response elicitor HreX increased the growth of Roundup® treated plants by 9 to 10%.

31. In order to investigate whether treatment of plants with the hypersensitive response elicitor HreX from *Xanthomonas campestris* imparts salt stress resistance in plants, lima bean seeds (Dixie Speckled Peas) were treated with HreX, sown, and then maintained in the presence of varying concentrations of NaCl. HreX treatment consisted of soaking the seeds in 100 ml of a solution containing a 3% formulation of HreX dissolved in water. The seeds were soaked for approximately 4 hours at 26°C. The lima beans were grown in pots containing vermiculite, equal amounts of the fertilizer EcoGrow (ECO Enterprises, Shoreline, WA), and varying concentrations of NaCl. The treatment groups were as detailed below in Figure 2. Fifteen seeds were planted in each pot, with a

total of six pots per treatment. Plants were grown at 22°C to 26°C with a 14 hour daylight period. Results were obtained by measuring the dry weight from the largest 10 plants from each pot. The plants were dried by isolating the entire plant from the vermiculate and drying overnight at 26°C. The Combined Weights detailed in Figure 2 below represent the accumulated dry weight of the 60 plants measured from each treatment. The untreated control (UTC) plants were not treated with HreX and were not grown in the presence of NaCl. The results of the study are shown below in Figure 2.

Figure 2. Dry Weight of Salt + HreX and Salt Alone Treated Plants



32. The hypersensitive response elicitor HreX clearly imparts salt stress resistance in plants, as demonstrated in Figure 2. Growth of the plants in the presence of 100 mM and 200 mM NaCl resulted in decreases in plant dry weight of approximately 38% and 57%, respectively, in comparison to that of the untreated control plants. In contrast, plants treated with HreX and grown in the presence of 100 mM and 200 mM NaCl resulted in decreases in plant dry weight of approximately 14% and 48%, respectively, in comparison to that of the untreated control plants. The treatment of plants growing in the presence of high concentrations of NaCl with the hypersensitive response elicitor HreX resulted in increases in plant dry weight of 18 to 28%.

33. I hereby declare that all statements made herein of my own knowledge are true and that all statements made on information and belief are believed to be true; and further that these statements were made with the knowledge that willful false statements and the like so made are punishable by fine or imprisonment, or both, under section 1001 of Title 18 of the United States Code, and that such willful false statements may jeopardize the validity of the application or any patent issuing thereon.

Date: Feb. 3/03

Zhong-Min Wei